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**Spatial variation of active microbiota in the rice rhizosphere revealed by
in situ stable isotope probing of phospholipid fatty acids**

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Abstract

This report is part of a serial study applying stable isotope labeling to rice microcosms to track the utilization of recently photosynthesized carbon by active microbiota in the rhizosphere. The objective of the present study was to apply phospholipid fatty acid-based stable isotope probing (PLFA-SIP) to detect the spatial variation of active microorganisms associated with rhizosphere carbon flow. In total, 49 pulses of $^{13}\text{CO}_2$ were applied to rice plants in a microcosm over a period of 7 d. Rhizosphere soil was separated from bulk soil by a root bag. Soil samples were taken from rhizosphere and bulk soil, and the bulk soil samples were further partitioned both vertically (up layer and down layer) and horizontally with increasing distance to the root bag. Incorporation of ^{13}C into PLFAs sharply decreased with distance to the roots. The labeling of 16:1 ω 9, 18:1 ω 7, 18:1 ω 9, 18:2 ω 6,9 and i14:0 PLFAs was relatively stronger in the rhizosphere while that of i15:0 and i17:0 increased in the bulk soil. The microorganisms associated with 16:1 ω 9 were active in both up- and down-layer soils. The microorganisms represented by i14:0, 18:1 ω 7 and 18:2 ω 6,9 exhibited a relatively higher activity in up layer soil, whereas those represented by i15:0 and i17:0 were more active in down layer soil. These results suggest that in the rhizosphere Gram-negative and eukaryotic microorganisms were most actively assimilating root-derived C, whereas Gram-positive microorganisms became relatively more important in the bulk soil. The active populations apparently differed between up- and down-layer soil and in particular changed with distance to the roots, demonstrating systematic changes in the activity of the soil microbiota surrounding roots.

1 **Introduction**

2 Rhizosphere carbon flow is of central importance in C cycling in terrestrial ecosystems.
 3 Isotope tracer techniques have been taken as useful approaches to investigate rhizosphere
 4 carbon flow in various soils. Using such technology, studies have revealed the influences of
 5 plant, soil, nutrition, climate condition and increased CO₂ on rhizodeposition (Kuzyakov
 6 and Domanski, 2000). The consensus estimate shows that 30 – 60% of the net
 7 photosynthesized C is allocated to roots, and as much as 40 – 90% of this fraction enters soil
 8 in the forms of rhizodeposits (Lynch and Whips, 1990). Most of the root-derived material is
 9 readily degradable by soil microbiota. However, little is known about the microbiota
 10 involved in the processing of root-derived C in soils.

11 The incorporation of root-derived C into microbial biomass has traditionally been
 12 investigated using a combination of isotope labeling and chloroform fumigation methods
 13 (e.g., Lu *et al.*, 2002; Rangel-Castro *et al.*, 2005a). Experiments using these techniques
 14 showed in various soils that the net incorporation of C into microbial biomass was
 15 approximately 0.8 – 5% of total photoassimilate (Liljeroth *et al.*, 1990; Martin and Merckx
 16 1992; Kuzyakov *et al.* 2000; van Ginkel *et al.*, 2000). In rice soil, it was estimated that on
 17 average 0.54% of the photoassimilated C was incorporated into microbial biomass over the
 18 growing season of the plants (Lu *et al.*, 2002). Although this is only a small proportion of
 19 the total photoassimilated C, the contribution accounts for 28% of total microbial biomass
 20 in soil. Moreover, the incorporation of the photosynthesized carbon was rapid (within 1 h
 21 after the pulse labeling of rice with ¹³CO₂) (Lu *et al.*, 2002) and methane was actively
 22 produced from root-derived C (Minoda and Kimura, 1994; Minoda *et al.*, 1996;

Dannenberg and Conrad, 1999). However, with traditional methodology, it is difficult to determine how individual microorganisms perform and contribute to C dynamics in soil.

Recently, stable isotope probing (SIP) has been used as a useful approach to link microbial identities with specific biogeochemical process in situ (Dumont and Murrell, 2005; Friedrich, 2006; Madsen, 2006; Whiteley *et al.*, 2006). This method has been also applied to track the utilization of root-derived C by active members in the rhizosphere (Prosser *et al.*, 2006). Phospholipid fatty acids (PLFA)-based SIP analysis showed that fungi and Gram-negative bacteria were most active in assimilating root-derived C in ryegrass soils (Butler *et al.*, 2003; Treonis *et al.*, 2004), and that microorganisms with straight and mono-unsaturated PLFAs were most active in rice soil (Lu *et al.*, 2004). To obtain a better resolution of the individual microbial groups, several studies have attempted using RNA-SIP approaches (Griffiths *et al.*, 2004; Rangel-Castro *et al.*, 2005b; Lu and Conrad, 2005; Lu *et al.*, 2006). Our previous studies in rice soil indicate that uncultured Rice Cluster I archaea, *Azospirillum* spp. and members of *Burkholderia* were most active in mediating rhizosphere C dynamics and CH₄ production (Lu and Conrad, 2005; Lu *et al.*, 2006).

However, the rhizosphere of plants is a dynamic environment and the niche condition in the vicinity of roots is highly variable with root growth. Gradients of physical, chemical and biological conditions are the rule in rhizosphere and bulk soil (Wind and Conrad, 1997; Ratering and Schnell, 2000; Young and Crawford, 2004; Noll *et al.*, 2005). Understanding how microbial communities respond to environmental gradients is crucial for predicting and managing the biogeochemical processes. The objective of the present study was to address

the spatial variation of activity and function of microbiota in rice rhizosphere. We have previously identified active archaeal and bacterial members that are involved in rhizosphere C dynamics (Lu and Conrad, 2005; Lu et al, 2006). In the present study, PLFA-SIP was applied to determine how active members varied spatially in rhizosphere and adjacent bulk soil using material from the previous labeling experiment (Lu and Conrad, 2005).

Results

Analysis of total PLFAs

PLFAs were analyzed in rhizosphere soil (inside root bag) and bulk soil of rice microcosms. Bulk soil was sampled at different distances to the root bag using horizontal soil cores inserted through the up-layer (1-4 cm depth) and down-layer (5-8 cm depth) soil. Principle Component Analysis (PCA) revealed a remarkable difference in the composition of total PLFAs between the rhizosphere and the bulk soil samples (Fig. 1). In particular, the poly-unsaturated and mono-unsaturated PLFAs were significantly higher, while the branched PLFAs were lower in the rhizosphere compared to the bulk soil samples (data not shown). The community composition in the bulk soil samples also showed a systematic change with distance to the roots at the level of the second principle component (Fig. 1). However, PLFA patterns in the bulk soil did not differ between up and down layers. Samples collected at a distance of >2 cm from the root surface formed a tight cluster regardless of soil depth (Fig. 1).

1 *Analysis of ^{13}C incorporation into PLFAs*

2 The $\delta^{13}\text{C}$ values of all the PLFAs detected in rice rhizosphere substantially increased
 3 over the 7-d period of rice labeling with $^{13}\text{CO}_2$ (Fig. 2A). However, the increase differed
 4 greatly among the individual PLFAs. The poly-unsaturated 18:2 ω 6,9 showed the highest
 5 increase in $\delta^{13}\text{C}$ ($\Delta\delta^{13}\text{C} = +8322\text{‰}$), followed by the mono-unsaturated 16:1 ω 9, 18:1 ω 7,
 6 18:1 ω 9, branched i14:0 and saturated 16:0 (Fig. 2A). The $\Delta\delta^{13}\text{C}$ values of the branched
 7 a15:0, i15:0, i16:0, a17:0 and i17:0 were only 30 – 40% of those of mono-unsaturated
 8 PLFAs. The 20:0 and 10Me18:0 showed the least $\Delta\delta^{13}\text{C}$ values. The $\delta^{13}\text{C}$ value of PLFAs
 9 extracted from bulk soils also increased, but to a remarkably smaller extent compared with
 10 the rhizosphere (Fig. 2A). The pattern of ^{13}C incorporations into PLFAs also differed
 11 between bulk and rhizosphere soil. The labeling index showed that the relative ^{13}C -labeling
 12 of 18:1 ω 9, 18:2 ω 6,9, i14:0 and 10Me18:0 was lower, while that of 16:1 ω 9 and most
 13 branched PLFAs (except i14:0) was higher in the bulk compared to the rhizosphere soil (Fig.
 14 2B). In particular, the labeling index of the branched i15:0 and i17:0 increased from 0.5 to
 15 2.2 and from 0.3 to 1.8 in the first section of the bulk soil versus the rhizosphere soil.

16 Net incorporation of ^{13}C into PLFAs sharply decreased with soil depth (Fig. 3A). The
 17 $\Delta\delta^{13}\text{C}$ values were between +37‰ to +1381‰ in the first section (relative to the root bag)
 18 of the up-layer soil but were only +0.3‰ to +301‰ in the first section of the down-layer
 19 soil. The 16:1 ω 9 received the highest ^{13}C labeling, followed by i15:0 and i17:0 PLFAs in
 20 the first section of up-layer soil (Fig. 3A). The labeling index showed that the relative
 21 labeling of 16:1 ω 9, i15:0 and i17:0 decreased while that of 18:1 ω 7, 18:1 ω 9, 18:2 ω 6,9,
 22 i14:0 and a15:0 increased in the down-layer soil compared with the up-layer soil (Fig. 3B).

23 To observe the differences in microbial activities at different distances to root surface,

the patterns of ^{13}C labeling were compared across 5 sections of the two horizontal soil cores. In the up-layer soil, both 16:1 ω 9 and 18:2 ω 6,9 were labeled to a relatively higher extent than i15:0 and i17:0 in the first section (0 – 6 mm) (Fig. 4A). The ^{13}C labeling of these PLFAs decreased with increasing distance to root. Other PLFAs showed relatively low labeling in the first section and decreased either gradually (18:1 ω 7 and i14:0) or steeply (a15:0, i16:0 and a17:0) with distance to root. Nevertheless, the incorporation of ^{13}C into 16:1 ω 9, 18:2 ω 6,9, 18:1 ω 7 and i14:0 still remained at a relatively high level in the second and third section (12 – 18 mm) of up-layer soil. Similarly, the labeling of most PLFAs in down-layer soil decreased with increasing distance to roots (Fig. 4B). But the ^{13}C incorporations into 16:1 ω 9, i15:0 and i17:0 remained at a relatively high level even in the second and third sections. Notably, the ^{13}C labeling of 18:2 ω 6,9 was well detectable in the third section of up-layer soil, but it decreased to virtually zero in the second section of down-layer soil.

Discussion

We conducted stable isotope labeling experiments in rice microcosms to identify microbial populations responsible for the processing of the recently-photosynthesized, root-derived C. Both RNA-SIP and PLFA-SIP approaches were applied to identify the active organisms associated with the rhizosphere carbon dynamics and methane production. In previous reports, we showed that some archaeal and bacterial members assimilated sufficient ^{13}C so that their RNA could be efficiently separated from those of less- or non-labeled organisms by density gradient centrifugation (Lu and Conrad, 2005; Lu *et al.*, 2006).

In the present study, we show that some microbial PLFAs were indeed highly labeled. The net ^{13}C incorporation resulted in ^{13}C enrichments of 16:1 ω 9; 18:1 ω 7,9; 18:2 ω 6,9; and i14:0 of about 6 – 9 atom% (calculated from $\delta^{13}\text{C}$ values) over 7 d labeling. Note, archaeal lipids were not targeted by the assays used. Furthermore, it is difficult to predict which bacterial taxa were represented by the detected PLFAs, as different bacterial species can have a similar PLFA composition (O’Leary and Wilkinson, 1988; Wilkinson, 1988). Nevertheless, the significant labeling of PLFAs demonstrates that some microorganisms did accumulate rather high amounts of ^{13}C into their biomass during the experiment. Since a relatively broad range of different phylogenetic groups share the same individual PLFA, it is likely that some of these groups had been active and incorporated high amounts of ^{13}C into their biomass, whereas other were inactive and did not incorporate ^{13}C . An individual PLFA thus may represent a mixture of active and inactive microbial groups, so that <9 atom% detected in particular PLFAs may in fact represent a mixture of microbial groups with both much higher and much lower labeling. Only the former would be sufficiently enriched in ^{13}C to allow successful separation of labeled nucleic acids by density centrifugation (DNA/RNA-SIP), e.g. the *Azospirillum* spp. and members of *Burkholderia* detected in our previous study (Lu *et al.* 2006; see below).

The PLFA profile differed greatly between rhizosphere and bulk soil (Fig. 1). This result is consistent with previous studies using culture-independent DNA or RNA fingerprinting. It was shown that *Proteobacteria*, particularly Alpha-, Beta- and Gamma-*proteobacteria* were dominant in the vicinity of rice roots (Lu *et al.*, 2006), whereas *Clostridium*, *Bacteroidetes*, *Verrucomicrobia*, and *Actinobacteria* were dominant

in the bulk soil (Hengstmann *et al.*, 1999; Lüdemann *et al.*, 2000). In various upland soil systems, it has also been demonstrated that structure and function of the microbiota in the rhizosphere are different from those in the adjacent bulk soil (Steer and Harris, 2000; Kowalchuk *et al.*, 2002; Söderberg *et al.*, 2002). Apparently, the releases of root exudates and the unique physicochemical conditions in the vicinity of roots cause a differentiation of the microbiota in rhizosphere versus bulk soil (Bolton *et al.*, 1993).

All mono- and poly- unsaturated PLFAs but only one branched PLFA (i14:0) were significantly labeled in rice rhizosphere (Fig. 2). Mono-unsaturated, poly-unsaturated, and branched PLFAs are indicative of Gram-negative, eukaryotic, and Gram-positive organisms, respectively (O’Leary and Wilkinson, 1988; Wilkinson, 1988; Frostegard and Baath, 1996). According to our PLFA data, Gram-negative organisms (represented by 16:1 ω 9, 18:1 ω 7 and 18:1 ω 9) and to some extent also Gram-positive organisms (represented by i14:0) actively utilized ¹³C-labeled rhizodeposits in rice rhizosphere. This result agrees well with the previous finding that straight and mono-unsaturated PLFAs 16:1 ω 9, 18:1 ω 7 and 18:1 ω 9 were associated with microorganisms that most actively assimilated the root-released carbon in a Japanese rice soil (Lu *et al.*, 2004). In anoxic environments, mono-unsaturated PLFAs may also represent Gram-positive organisms such as *Clostridium* spp. (Lu *et al.*, 2004). Nevertheless, RNA-SIP in the same soil revealed that *Azospirillum* spp. and members of *Burkholderia* were most active in utilizing root exudates in rice rhizosphere (Lu *et al.*, 2006). Both groups are Gram-negative and contain mainly mono-unsaturated PLFAs (Wilkinson, 1988). It is, therefore, probable that the labeling of 16:1 ω 9, 18:1 ω 7 and 18:1 ω 9 reflected the activities of these organisms in rice rhizosphere, although the highest labeled

PLFA do not fit perfectly to those reported from *Burkholderia* isolates (Tillmann *et al.*, 2005).

Although precautions were taken to remove living root material from soil prior to PLFA extraction, minor contamination by root cells could not be completely excluded. This might cause overestimation of ^{13}C incorporation into eukaryotic PLFAs, particularly the 18:1 ω 9 and 18:2 ω 6,9 which are present in plant cells. Nonetheless, labeled 18:2 ω 6,9 was also clearly detected in bulk soil, which was free of roots due to separation by root bags. Thus, eukaryotic microorganisms containing 18:2 ω 6,9 probably also played an important role in processing root-derived C in soil. Studies in grassland soil also showed that the eukaryotic organisms associated with 18:2 ω 6,9 were the most active in utilizing root-derived C (Butler *et al.*, 2003; Trenios *et al.*, 2004). Clearly, eukaryotic microorganisms, most probably fungi, are active in metabolizing the root-derived C in various soil ecosystems.

Outside the root bags, the ^{13}C labeling of PLFAs sharply decreased (Fig. 2A). Furthermore, the relative labeling of mono- and poly- unsaturated PLFAs (16:1 ω 9, 18:1 ω 7, 18:1 ω 9 and 18:2 ω 6,9) significantly decreased. The extent of ^{13}C labeling of PLFAs also decreased rapidly with increasing distance to the roots (Fig. 4). However, the labeling index of branched PLFAs (i15:0 and i17:0) increased in the bulk soil relative to the other PLFAs (Fig. 2B). Apparently, most of root-derived C was rapidly metabolized by the microbial community in the rhizosphere, particularly by Gram-negative (16:1 ω 9, 18:1 ω 7 and 18:1 ω 9) and eukaryotic (18:2 ω 6,9) microorganisms. Although less of the photosynthate C was assimilated by microorganisms in the bulk compared to the rhizosphere soil, this activity

was dominated by Gram-positive microorganisms associated with i15:0 and i17:0.

There was not much difference in total PLFAs between up- and down-layer soils (Fig. 1), but net ^{13}C incorporation into PLFAs markedly decreased in down-layer compared with up-layer soil (Fig. 3A). The 16:1 ω 9, 18:1 ω 7, i14:0 and 18:2 ω 6,9 exhibited relatively higher labeling in up-layer soil, while labeling of 16:1 ω 9, i15:0 and i17:0 relatively dominated in down-layer soil. Apparently, the respective microorganisms became active in up-layer and down-layer soils. These differences were likely due to changes in quantity and quality of root-derived C and soil physicochemical conditions with soil depth (Kirk, 2003).

Conclusions

Using PLFA-SIP, the present study showed that Gram-negative (represented by 16:1 ω 9, 18:1 ω 7, 18:1 ω 9) and eukaryotic (18:2 ω 6,9) microorganisms were the most active ones in assimilating root-derived C in rice rhizosphere, while Gram-positive microorganisms (i14:0, i15:0 and i17:0) were relatively more important in the bulk soil. So far, few studies have considered the spatial variation of microbial communities in soil. Using PLFA-SIP analyses, the present study demonstrates that the activity of microbial communities indeed changes vertically and horizontally in rice soil. Although, the PLFA analyses were similar for the up-layer and down-layer soil of our rice microcosms, we presently do not know how reproducible these data might be for other combinations of rice cultivars and soil types. Determination of such variations shall prove being essential for a better understanding of C cycle in soils.

Experimental procedures

Rice growth and ^{13}C labeling

The preparation of microcosms and the labeling procedures with ^{13}C have been explained in detail in previous reports (Lu and Conrad, 2005; Lu *et al.* 2006). In brief, soil sample was taken from wetland rice fields at the Italian Rice Research Institute in Vercelli, Italy. The soil properties have been described before (Schütz *et al.*, 1989a; 1989b). Rice was directly seeded into a nylon mesh bag, which was placed into the middle of a soil container (20 × 10 × 13 cm) containing 3.5 kg of soil (dw). The function of mesh bags was to keep the growing roots within a relatively smaller volume (soil inside root bag was defined as rhizosphere), while nutrients were allowed to pass freely for plant uptake (Lu *et al.*, 2000). The soil containers were prepared following the description by Wind and Conrad (1997). They consisted of two matching counterparts with four holes (i.d. = 3 cm) at 4 and 8 cm depth on two opposite sides. The holes were sealed with rubber stoppers during plant growth and used for taking horizontal soil cores at the end of the experiment. The soil was fertilized with 0.28 g N, 0.14 g K and 0.07 g P kg⁻¹ soil and flooded throughout the growing period. Labeling with ^{13}C was carried out at 45 d after rice seeding in a 10-L top closed chamber. In total, 49 pulses of $^{13}\text{CO}_2$ were supplied to rice plants over a 7-d period. The $^{13}\text{CO}_2$ (99 atom ^{13}C %) was generated through reaction between $\text{NaH}^{13}\text{CO}_3$ (Cambridge Isotope Laboratory) and H_3PO_4 in a serum vial. Parallel microcosms were established without ^{13}C labeling as controls.

Soil sampling

At the end of labeling, plastic soil core samplers were horizontally inserted through holes on two opposite sides of soil container. Cores were inserted at two soil depths, up layer (1-4 cm) and down layer (5-8 cm). The cores were fixed by pressing the opposite cores against each other. The two parts of soil container was then taken apart and the horizontal soil cores were taken out. The soil core samples (bulk soils) were immediately frozen in liquid N₂ and stored at -70°C. Roots with adhering soil were removed from the root bags. Soil and roots were separated by carefully washing with de-ionized water. The soil slurries were collected, mixed thoroughly, and centrifuged at 3000 rpm. The floating root material and supernatants were disposed and the remaining soil (rhizosphere soil) was immediately frozen and stored at -70°C.

PLFA extraction and ¹³C profiling

Prior to PLFA extraction, soil cores were thawed and cut into 5 sections of 6 mm thickness each using a sterile stainless steel knife at 4°C. Total PLFA was extracted from 5 g wet soil samples using the Bligh-Dyer method with minor modification (Knief *et al.*, 2003). The extracted lipids were separated on a solid phase extraction column (Sep-Pack[®] Cartridges Silica, Waters) (Lu *et al.*, 2004) and the fatty acid components were released and methylated by mild alkaline methanolysis (White *et al.*, 1979). The individual fatty acid methyl esters (FAMES) were identified using GC-MS and their stable carbon-isotope compositions were determined using a Finnigan MAT Model 252 isotope ratio mass spectrometer coupled to HP 5890 gas chromatograph with a Finnigan standard GC combustion interface (Abraham *et al.*, 1998). The stable isotope ratios of individual FAMES

were corrected for the isotope composition of the exogenous methyl derivative group to obtain the actual ratios of PLFAs (Rieley *et al.*, 1994). Total PLFAs were estimated based on peak areas in gas chromatographs with 19:0 as internal standard. Standard nomenclature is used to describe PLFAs (Butler *et al.*, 2003). The net ^{13}C labelling of PLFAs was calculated as $\delta^{13}\text{C}$ values of PLFAs in the labelled samples in excess of those in non-labelled control, i.e.

$$\Delta\delta^{13}\text{C} = \delta^{13}\text{C}_{\text{labeled}} - \delta^{13}\text{C}_{\text{non-labeled}}$$

The relative labeling was calculated by normalizing the ^{13}C incorporation (increase of ^{13}C atom%) into the PLFA against the PLFA 16:0, which was set to unity in each sample. This PLFA was chosen as it is ubiquitous in various organisms. The relative labeling (labeling index) of individual PLFAs was estimated by comparing the net ^{13}C labeling of each PLFA with that of 16:0.

One microcosm each for ^{13}C -labeling and control were destructed for soil sampling and PLFA extraction. Two extracts at same positions of the opposite sides for the bulk soil were combined prior to PLFA analysis. Hence, data represent means of duplicate samples for the bulk soils. Total PLFA compositions of all samples were subjected to principle component (PC) analysis (PCA) using the software SPSS 12.0 for Windows.

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Captions of Figures

Fig. 1. Principle component analysis of total PLFAs in soil from non-labeled (control) and labeled microcosms, which were separated into rhizosphere soil (R), and bulk soil's up-layer (1 – 4 cm) and down-layer (5 – 8 cm). The “a – e; I – V” indicate five sections (6 mm thickness) of control, up-layer and down-layer core samples, respectively.

Fig. 2. Incorporation of ^{13}C into individual PLFAs extracted from rhizosphere soil (“R”) and the first section of the up-layer core [“B-up(0-6 mm)”]. (A) Net increase of $\delta^{13}\text{C}$ values of PLFAs in the labelled samples in excess of those in the non-labelled control; (B) labeling index calculated by normalizing to the ^{13}C incorporation (increase of ^{13}C atom%) into PLFA 16:0.

Fig. 3. Incorporation of ^{13}C into individual PLFAs in up- and down-layer bulk soil samples, “B-up(0-6 mm)” and “B-down(0-6 mm)” indicate the first section of the up-layer and down-layer core, respectively. (A) Net increase of $\delta^{13}\text{C}$ values of PLFAs in the labelled samples in excess of those in the non-labelled control; (B) labeling index calculated by normalizing to the ^{13}C incorporation (increase of ^{13}C atom%) into PLFA 16:0.

Fig. 4. Change of net ^{13}C incorporation into PLFAs (expressed as $\Delta\delta^{13}\text{C}$) with increasing distance to the root surface. (A) Up-layer soil core (1 – 4 cm from soil surface); (B) down-layer soil core (5 – 8 cm from soil surface).

Figure 1

Figure 2

Figure 3

Figure 4